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Decreased IL-2, IFN-γ, and IL-10 Production by Aged Mice During the Acute Phase of E55+ Retrovirus Infection

Mohamed Elrefaei,* Kenneth J. Blank,*,† and Donna M. Murasko*,[1]

*Department of Microbiology and Immunology, MCP Hahnemann University School of Medicine, Philadelphia, Pennsylvania; and †Department of Pathology and Laboratory Medicine, MCP Hahnemann University School of Medicine, Philadelphia, Pennsylvania

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We previously reported that aged mice demonstrated a 12-week delay in virus clearance compared to young mice after infection with E55+ murine leukemia retrovirus (E55+MuLV). The current study demonstrates that both the levels of IL-2, IFN-γ, and IL-10 and the number of cells producing IL-2 and IFN-γ were lower at 2 and 4 weeks postinfection (p.i.) in aged compared to young mice after virus-specific stimulation of spleen cells *in vitro.* In both age groups, IL-2 and IL-10 were produced by $CD4^+$ T and B cells, respectively. IFN-γ was produced mainly by $CD4^+$ T cells at 2 weeks p.i. and by $CD4^+$ and $CD8^+$ T cells at 4 weeks p.i. in young, but primarily by $CD8^+$ T cells, in aged mice. Therefore, delayed virus clearance is associated with age-related decreases in type 1 and type 2 cytokines and a shift in the primary source of at least one cytokine.

Key Words: aging; retrovirus; IL-2; IFN-γ; IL-10; $CD4^+$ T cell; $CD8^+$ T cells; B cell.

INTRODUCTION

Aging is associated with an increased incidence of virus infections (Schneider, 1983; Louria *et al.,* 1993). This increase has been attributed to age-related alterations in both T cell mediated (Kirschmann and Murasko, 1992; Goonewardene and Murasko, 1993; Chakravarti and Abraham, 1999; Pawelec *et al.,* 1999) and humoral (LeMaoult *et al.,* 1997; Song *et al.,* 1997) immunity. The age-releated decrease in immune function is also associated with significant alterations in cytokine production. Numerous studies have shown that a decrease in IL-2 production occurs with aging (Kirschmann and Murasko, 1992; Goonewardene and Murasko, 1993; Engwerda *et al.,* 1996; Wakikawa *et al.,* 1999). Further, a limited number of studies has shown an age-related increase in IL-10 production (Hobbs *et al.,* 1994; Spencer *et al.,* 1996). While it has been postulated that aging is associated with an increase in type 2 cytokines and a decrease in type 1 cytokines, some studies have demonstrated an age-associated increase in IFN-γ (Kirschmann and Murasko, 1992; Goonewardene and Murasko, 1993; Engwerda *et al.,* 1996; Wakikawa *et al.,* 1999) and others a decrease in IFN-γ (Frasca *et al.,* 1997; Fujo *et al.,* 1995; Taylor *et al.,* 1997). In the majority of these cytokine studies a polyclonal activator (e.g., concanavalin A (Con A), anti-CD3 antibody) was used to stimulate cytokine production. Only a limited number of studies has examined the effect of aging on antigen-specific cytokine production (Frasca *et al.,* 1997), and, importantly, the effect of aging on cytokine production induced specifically by a virus during infection has not been directly examined.

E55+ murine leukemia virus (E55+MuLV) is a replication-comptetent murine retrovirus that causes a chronic infection, but fails to cause leukemia, in C57BL strains of mice (Avidan *et al.,* 1995). Infection of C57BL/6 (B6) mice is characterized by an acute and a persistent phase. The acute phase is characterized by the presence of large numbers of virus-infected cells in the spleen and bone marrow during the first 2–4 weeks postinfection (p.i.). The number of virus-infected cells decreases to undetectable levels by 8 weeks p.i. (Panoutsakopoulou *et al.,* 1998). However, the virus is not eradicated, with infected cells remaining sequestered in lymphoid tissue during the persistent phase of infection (Avidan *et al.,* 1995; Panoutsakopoulou *et al.,* 1998; Tumas *et al.,* 1993). The antivirus immune response has been demonstrated to play an important role in controlling virus infection during the acute phase because mice sublethally irradiated prior to inoculation with E55+MuLV demonstrate no clearance of virus (Avidan *et al.,* 1995; Panoutsakopoulou *et al.,* 1998). We have previously reported that unlike 6-month-old (young) B6 mice that decreased E55+MuLV burden to undetectable levels by 8 weeks p.i., the virus burden in 22-month-old (aged) mice did not reach undetectable levels until 20 weeks p.i. (ElRefaei *et al.,* 2001). This delay was not the result of enhanced virus replication in aged mice: kinet-

[1] To whom correspondence and reprint requests should be addressed at Department of Microbiology and Immunology, MCP Hahnemann University School of Medicine, 2900 Queen Lane, Philadelphia, PA 19129. Fax: (215) 848-2271. E-mail: donna.murasko@drexel.edu.

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ics of virus replication, peak titers, and tissue distribution were comparable in young and aged mice (ElRefaei *et al.,* 2001). However, the delay in virus clearance was associated with the lack of a T cell proliferative response and significantly lower cytotoxic T cell response and virus neutralizing antibody titers to E55+MuLV in aged compared to young B6 mice (ElRefaei *et al.,* 2001).

In the present studies we utilized the E55+MuLV model to directly examine the effect of age on cytokine production during virus infection. Cytokines were examined at 2 weeks p.i., the time of the initiation of a detectable T cell response, and at 4 weeks p.i., the time of peak T cell response. All of the cytokines detected were significantly reduced in age compared to young mice. The lower levels of cytokines in aged mice were associated with a decreased number of cells producing the cytokines. With one cytokine the subset of T cells producing the cytokine was also different in aged compared to young mice. These results demonstrate that the observed delay in E55+MuLV clearance by aged mice is associated with significant alterations in both type 1 and type 2 cytokines.

RESULTS

T cell proliferation and IL-2 production by young and aged mice

To directly examine age-associated alterations in T cell proliferation and cytokine production during E55+MuLV infection, groups of young and aged B6 mice were infected with E55+MuLV and then sacrificed either at 2 or 4 weeks p.i.

Spleen cells from young mice generated a significant proliferative response to E55+MuLV at 2 and 4 weeks p.i. (Fig. 1). In contrast, spleen cells from aged mice failed to generate a significant proliferative response at either time. $CD4^+$ T cells mediated the proliferative response of young mice because *in vitro* depletion of these cells prior to stimulation completely abolished proliferation. In contrast, *in vitro* depletion of the $CD8^+$ T cells had no effect on the response of either age group (data not shown).

To determine if the lack of proliferation by spleen cells of aged mice was associated with decreased IL-2 production, supernatants from *in vitro* cultures were tested by ELISA for the presence of IL-2. Culture supernatants of spleen cells from young mice cultured either alone or with uninfected IC21 cells contained significantly higher levels of IL-2 than culture supernatants of spleen cells from aged mice at 2 weeks p.i. ($P < 0.006$; Table 1). Although IL-2 levels increased significantly from 2 to 4 weeks in the culture supernatants of spleen cells from aged, but not young, mice ($P < 0.04$), IL-2 levels were still higher in culture supernatants of spleen cells from young mice at 4 weeks ($P < 0.04$). Contrary to our expectations, culture with E55+MuLV-infected IC21 cells

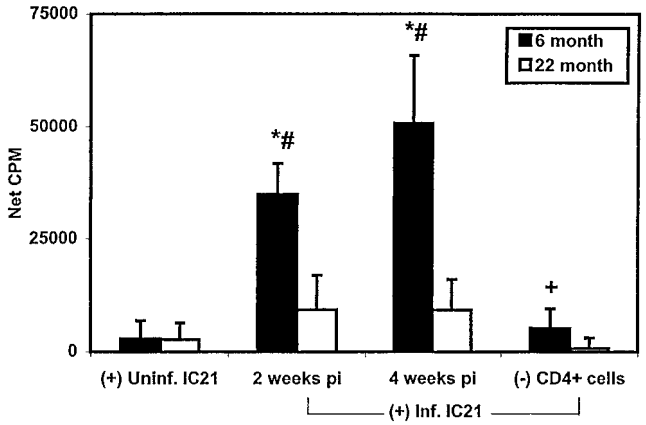

FIG. 1. T cell proliferative response to E55+MuLV of young and aged mice. Spleen cells from individual E55+MuLV-infected B6 mice at 2 and 4 weeks p.i. were cultured with either uninfected or E55+MuLV-infected IC21 cells. Aliquots of the spleen cells were depleted *in vitro* of either $CD4^+$ or $CD8^+$ T cells prior to culture. Proliferation is expressed as net counts per minute (Net CPM). Values are means ± SEM of a total of 9–12 mice/group evaluated in three separate experiments. Values of both the control group (spleen cells cultured with uninfected IC21 cells) and the $CD4^+$ cell-depleted group are means ± SEM of both times p.i. Statistically significant differences as determined by Student's *t* test: (*) Uninfected IC21 cells vs infected IC21 cells of the same age group, $P < 0.014$; (#) Young vs aged at the same time p.i., $P < 0.025$; (+) No depletion vs (−) $CD4^+$ cells of the same age group, $P < 0.014$. Differences between 2 and 4 weeks p.i. were not statistically significant.

resulted in a significant decrease in IL-2 levels relative to IL-2 levels of cultures with uninfected IC21 cells ($P < 0.02$) by spleen cells from young and aged mice at both times. However, IL-2 levels were still significantly higher in culture supernatants of spleen cells from young mice ($P < 0.04$).

To determine if the decrease in IL-2 levels in cultures of spleen cells from aged mice was associated with a decreased number of IL-2 producing cells, spleen cells cultured with E55+MuLV-infected IC21 cells were used in an indirect ELISPOT assay. Similar to the results of the ELISA, aged mice had significantly lower numbers of cells producing IL-2 than young mice at both 2 and 4 weeks p.i. In contrast to the results of the ELISA, virus-specific stimulation *in vitro* did not result in a significant decrease in the number of IL-2 producing cells in either age group compared to culture alone (data not shown) or with uninfected IC21 cells (Fig. 2A). In addition, there was no increase in the number of IL-2-producing cells at 4 compared to 2 weeks. $CD4^+$ T cells were the main source of IL-2 in both age groups because depletion of the $CD4^+$, but not $CD8^+$ (data not shown), cells prior to culture significantly decreased the level of IL-2 in the supernatant detected by ELISA (Table 1) and the number of IL-2-producing cells detected by inidrect ELISPOT (data not shown).

The contrasting data from the ELISA and the indirect ELISPOT suggested that *in vitro* culture has a major

TABLE 1

IL-2 Production by Young and Aged C57BL/6 Mice[a]

		Infected splenocytes cultured with		
		Uninfected IC21	Infected IC21	
Age	Weeks p.i.	Intact	Intact	(−) $CD4^+$
6 months	2	101 ± 21[b]	54 ± 11[b,c]	18 ± 2[d]
	4	136 ± 24[b]	76 ± 9[b,c]	22 ± 4[d]
22 months	2	26 ± 5	16 ± 2[c]	<15
	4	78 ± 17[e]	46 ± 10[c,e]	19 ± 2[d]

[a] Spleen cells from individual B6 mice were stimulated *in vitro* with IC21 cells. Culture supernatants were assayed by ELISA and results were expressed in pg/ml. Values are means ± SEM of nine individual mice/group evaluated in three separate experiments. Culture supernatants from infected spleen cells cultured either alone or with uninfected IC21 cells contained comparable levels of IL-2 (data not shown). Culture supernatants from infected spleen cells cultured with uninfected IC21 cells but depleted of $CD4^+$ T cells prior to culture contained <20 pg/ml of IL-2. Culture supernatant from naïve spleen cells contained below detectable levels of IL-2. Statistically significant differences as determined by Mann–Whitney *U* test:

[b] Young vs aged, $P < 0.006$ and $P < 0.04$ at 2 and 4 weeks p.i., respectively.

[c] IL-2 levels of uninfected IC21 cells vs infected IC21 cells of the same age group, $P < 0.02$.

[d] Intact vs (−) $CD4^+$ depletion of the same age group, $P < 0.005$.

[e] 2 weeks vs 4 weeks p.i. in aged mice, $P < 0.04$.

influence on the results obtained. To more closely reflect the *in vivo* situation, spleen cells from E55+MuLV-infected mice were used in direct ELISPOT assays. E55+MuLV infection resulted in detectable numbers of IL-2-producing cells in both age groups at 2 weeks ($P < 0.04$) that increased significantly by 4 weeks p.i. (Fig. 2B, $P < 0.03$). Consistent with both the ELISA and the indirect ELISPOT, the number of IL-2-producing cells was significantly higher in young compared to aged mice ($P < 0.05$) at both times. However, the number of IL-2-producing cells detected by direct ELISPOT was significantly lower than the numbers obtained by indirect ELISPOT in both age groups at both times ($P < 0.02$). These data suggest that *in vitro* culture of spleen cells either alone or with IC21 cells results in a significant increase in the number of IL-2-producing cells in both age groups. Importantly, data from all the assays indicated that $CD4^+$ T cells are responsible for IL-2 production in both age groups: depletion of $CD4^+$, but not $CD8^+$, cells prior direct ELISPOT assay decreased the number of IL-2-producing cells to levels comparable to those seen in cultures of naïve spleen cells (data not shown).

IFN-γ production by young and aged mice

Since the effect of aging on IFN-γ production during virus infection has not been clearly demonstrated, we evaluated IFN-γ production during the acute phase of E55+MuLV infection. The amount of IFN-γ in the supernatants of *in vitro* cultures that were assessed for proliferation was determined by ELISA. At 2 weeks p.i., eight of the nine young mice, while only two of the nine aged mice produced detectable IFN-γ levels (Fig. 3). At 4 weeks p.i., all the young mice tested (nine mice) had detectable IFN-γ, while only 8 of the 10 aged mice had detectable IFN-γ. Although the mean IFN-γ levels were not significantly higher at 4 compared to 2 weeks p.i. in

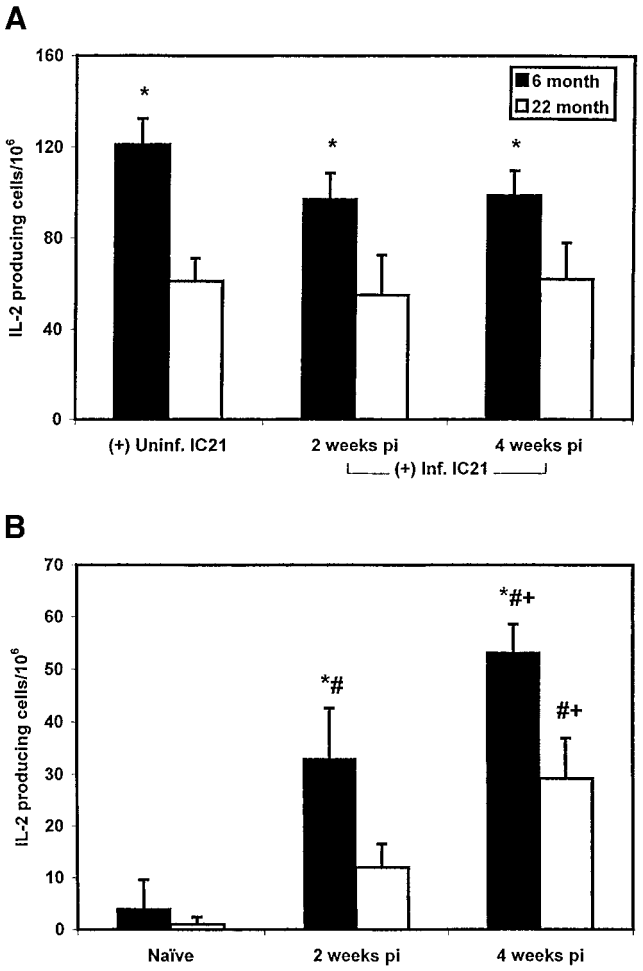

FIG. 2. Number of IL-2 producing cells following E55+MuLV-specific stimulation of spleen cells *in vitro*. Spleen cells from individual B6 mice were cultured either alone or with IC21 cells and then used in either indirect (A) or direct (B) ELISPOT assays. Data shown are means ± SEM of nine individual mice/group evaluated in three separate experiments. Values of the control group (spleen cells cultured with uninfected IC21 cells) are means ± SEM of both times p.i. For the indirect ELISPOT, spleen cells from infected mice of both age groups cultured either alone or with uninfected IC21 cells produced comparable numbers of IL-2-producing cells/10^6 cells. Naïve spleen cells cultured either alone or with IC21 cells produced <10 IL-2 producing cells/10^6 cells. Statistically significant differences as determined by Mann–Whitney *U* test: (*) Young vs aged at the same time p.i. and under similar conditions, $P < 0.02$; (#) Naïve vs infected of the same age group, $P < 0.04$; (+) 2 weeks vs 4 weeks p.i. of the same age group, $P < 0.03$. Differences between uninfected and infected IC21 cells of the same age group, and between 2 and 4 weeks p.i. of the same age group were not statistically significant for indirect ELISPOT (A).

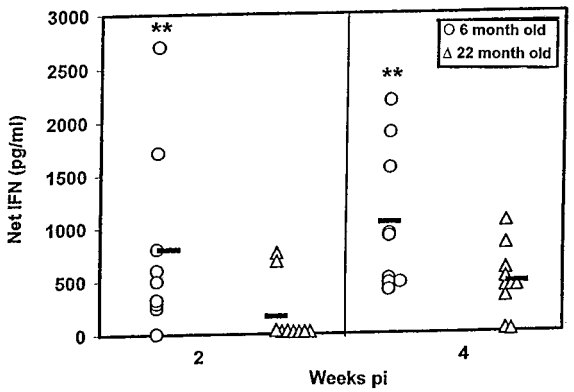

FIG. 3. IFN-γ production following E55+MuLV-specific stimulation of spleen cells *in vitro*. Spleen cells from B6 mice were cultured with either uninfected or E55+MuLV-infected IC21 cells. Culture supernatants were assayed by ELISA and results were expressed as net IFN-γ levels in pg/ml (Net pg/ml). Data are from 9–10 individual mice/group evaluated in three separate experiments. Bars represent the mean IFN-γ level/group. Culture supernatants of spleen cells from naïve B6 mice contained below detectable levels of IFN-γ. Statistically significant differences as determined by Mann–Whitney *U* test: (**) Young vs aged, $P < 0.03$ and $P < 0.04$ at 2 and 4 weeks p.i., respectively. Differences between 2 and 4 weeks p.i. were not statistically significant in either age group.

either age group, young mice had significantly higher mean IFN-γ levels than aged mice at both 2 ($P < 0.03$) and 4 ($P < 0.04$) weeks p.i. These results suggested that aged mice demonstrate a delay in the production of IFN-γ, as well as significantly lower mean IFN-γ levels compared to young B6 mice.

To determine whether the decreased IFN-γ production in aged mice was associated with a decreased number of IFN-γ producing cells, spleen cells cultured with E55+MuLV-infected IC21 cells were used in an indirect ELISPOT assay. At 2 weeks p.i., virus-specific stimulation resulted in a detectable number of IFN-γ-producng cells in only young mice (Fig. 4A). At 4 weeks p.i., IFN-γ-producing cells were detectable in both age groups; however, the number of IFN-γ producing cells was significantly higher at 4 compared to 2 weeks p.i. in young ($P < 0.01$), but not aged, mice (Fig. 4B). At both times, the number of virus-specific IFN-γ producing cells was significantly higher in young compared to aged B6 mice ($P < 0.02$). These results indicate that the lower IFN-γ level observed in supernatants was associated with significantly fewer IFN-γ-producing cells in the spleen of aged B6 mice. We were unable, however, to detect any IFN-γ-producing cells by direct ELISPOT.

To determine the contribution of T cell subsets to E55+MuLV-specific IFN-γ production in young and aged B6 mice, spleen cells were depleted of $CD4^+$ or $CD8^+$ T cells prior to culture. At 2 weeks p.i., depletion of the $CD4^+$ T cells of young mice decreased the number of E55+MuLV-specific IFN-γ-producing cells to levels comparable to those seen in culture of unstimulated spleen cells ($P < 0.04$; Fig. 4A). Depletion of the $CD8^+$ T cells, however, decreased the number of E55+MuLV-specific IFN-γ producing cells in only two of six mice, resulting in a mean 45% reduction compared to the undepleted controls. At 4 weeks p.i., depletion of either T cell subset in young mice resulted in a significant decrease in the number of IFN-γ producing cells ($P < 0.04$; Fig. 4B), with a reduction of 63 and 77% upon depletion of $CD4^+$ and $CD8^+$ T cells, respectively. These results suggest that about 25% of IFN-γ is directly produced by $CD4^+$ T cells. While 75% of the IFN-γ in young mice appear to be produced by $CD8^+$ T cells, about half of this seems to be dependent on the presence of both $CD4^+$ and $CD8^+$ T cells: Depletion of $CD4^+$ T cells results in a 60% reduc-

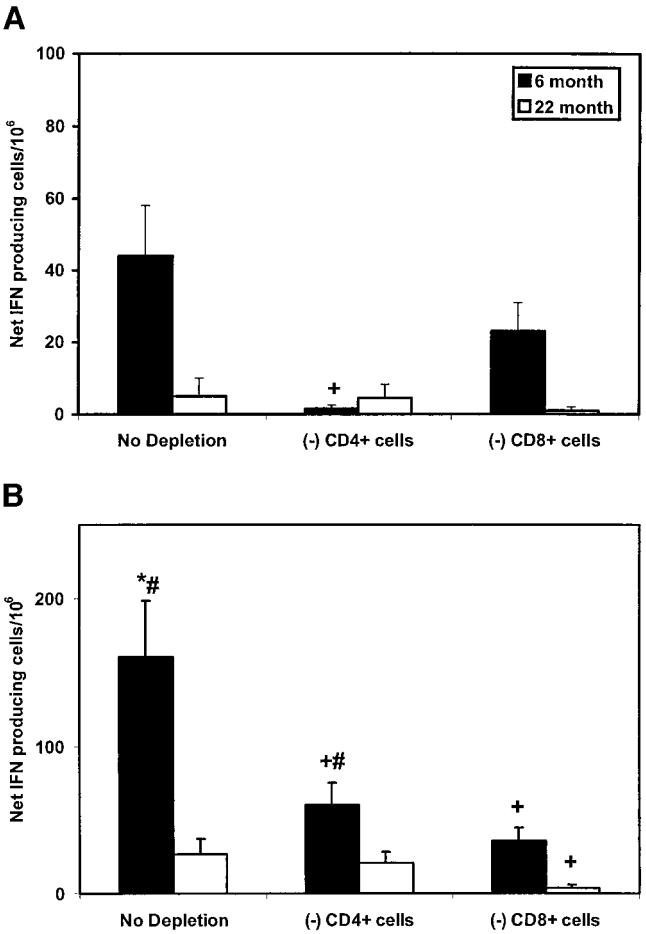

FIG. 4. Number of IFN-γ producing cells following E55+MuLV-specific stimulation of spleen cells *in vitro*. Spleen cells from individual B6 mice at 2 (A) and 4 (B) weeks p.i. were cultured with either uninfected or E55+MuLV-infected IC21 cells. Cells were then used in indirect ELISPOT assays and results were expressed as net IFN-γ producing cells/10^6 cells. Data shown are means ± SEM of nine individual mice/group evaluated in three separate experiments. Aliquots of the spleen cells were depleted of $CD4^+$ or $CD8^+$ T cells prior to culture. Naïve spleen cells cultured either alone or with IC21 cells produced <10 IFN-γ-producing cells/10^6 cells. Statistically significant differences as determined by Mann–Whitney *U* test: (*) Young vs aged at the same time p.i., $P < 0.02$; (+) No depletion vs (−) $CD4^+$ or (−) $CD8^+$ cells of the same age group and time p.i., $P < 0.04$; (#) 2 weeks vs 4 weeks p.i. of the same age group and under similar conditions, $P < 0.01$.

tion of IFN-γ-producing cells. However, since 25% of IFN-γ-producing cells are $CD4^+$ T cells, the remaining 35% reduction must be due to interactions of $CD8^+$ T cells with $CD4^+$ T cells.

In contrast, depletion of the $CD8^+$ T cells of aged mice resulted in a significant decrease (82%) in the number of IFN-γ-producing cells ($P < 0.04$), while depletion of $CD4^+$ T cells had no significant effect, only reducing the number of IFN-γ-producing cells by 22%. These results suggest not only that about 85% of the cells producing IFN-γ are $CD8^+$ T cells, but also that most of these $CD8^+$ T cells are capable of this production without help from $CD4^+$ T cells, as indicated by the congruence of $CD8^+$ and $CD4^+$ T cell depletion data (i.e., 18% of residual production by $CD4^+$ T cells in the former and a 22% reduction in the latter). In support of this conclusion, in 3 of 10 aged mice, $CD4^+$ T cell depletion had no effect on the number of cells producing IFN-γ. Similar results were observed by depletion of $CD4^+$ or $CD8^+$ T cells prior to culture of cells for the ELISA assay (data not shown). These results indicate that aged mice demonstrate an alteration in the phenotype of the cells that produce IFN-γ following E55+MuLV-specific stimulation of spleen cells *in vitro.*

IL-10 production by young and aged mice

Since aging is thought to be associated with an increase in type 2 cytokines such as IL-10 (Hobbs *et al.,* 1994; Spencer *et al.,* 1996), we assessed IL-10 production during the acute phase of E55+MuLV infection. At 2 weeks p.i., young mice demonstrated significantly higher levels of IL-10 ($P < 0.04$) in the supernatant of spleen cell cultures stimulated with E55+MuLV compared to unstimulated spleen cells (data not shown). Contrary to expectations, aged mice showed no significant increase in IL-10 levels upon virus-specific stimulation of spleen cells *in vitro* (Fig. 5A). Virus-specific stimulation resulted in significantly higher mean IL-10 levels at 4 compared to 2 weeks p.i. in both age groups ($P < 0.015$). However, supernatants of spleen cell cultures from young mice had significantly higher mean IL-10 levels than aged mice ($P < 0.05$; Fig. 5B). We tried to determine whether the decreased IL-10 production in aged mice was associated with a decreased number of IL-10-producing cells. However, we were unable to detect any IL-10 producing cells by direct or indirect ELISPOT assays in either age group possibly because the amount of IL-10 produced by the spleen cells was below the limit of detection of the assay.

To determine the source(s) of the IL-10, spleen cells were depleted of either $CD4^+$ or $CD8^+$ T cells or $CD19^+$ B cells prior to culture. At 2 weeks p.i., *in vitro* depletion of either T cell subset resulted in a 50% decrease in IL-10 levels in young B6 mice ($P < 0.05$). However, *in vitro* depletion of $CD19^+$ B cells resulted in reduction in IL-10 levels to background levels ($P < 0.02$; Fig. 5A). At 4 weeks p.i., *in vitro* depletion of $CD19^+$ cells resulted in a decrease in IL-10 to background levels ($P < 0.02$; Fig. 5B). Interestingly, depletion of $CD4^+$, but not $CD8^+$, T cells resulted in a 50% decrease in IL-10 production by spleen cells of young mice ($P < 0.05$), while depletion of either T cell subset had no effect on IL-10 production by spleen cells of aged mice.

Since IL-4 is another type 2 cytokine, we tried to determine the level of IL-4 in the culture supernatant, as well as the number of IL-4-producing cells, following E55+MuLV-specific stimulation of spleen cells from B6 mice. However, IL-4 levels were below detection in both age groups. These results may reflect the sensitivity of

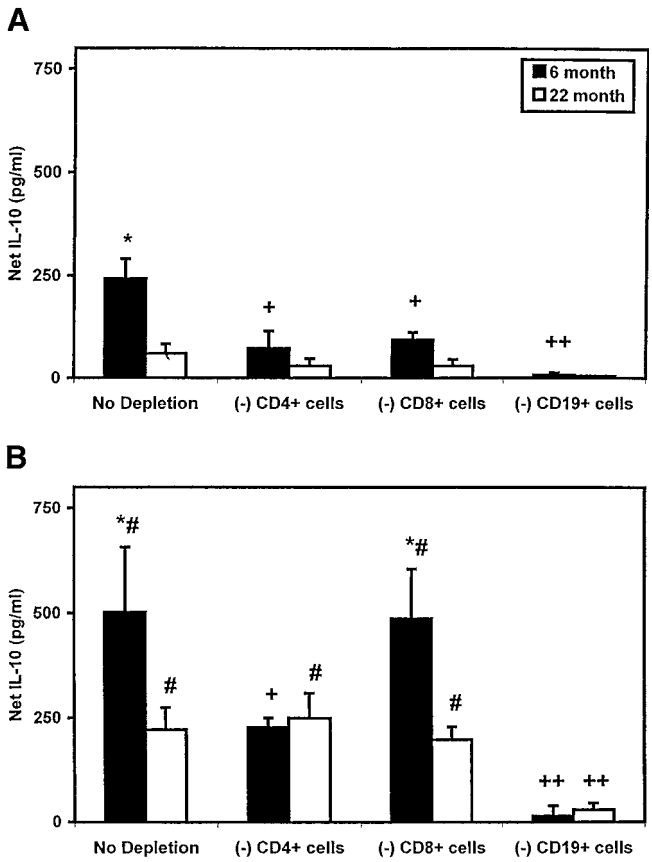

FIG. 5. IL-10 production following E55+MuLV-specific stimulation of spleen cells *in vitro.* Spleen cells from individual B6 mice at 2 (A) and 4 (B) weeks p.i. were cultured with either uninfected or E55+MuLV-infected IC21 cells. Culture supernatants were assayed by ELISA and results were expressed as net IL-10 levels in pg/ml. Data are means ± SEM of 9–12 individual mice evaluated in three separate experimetns. Aliquots of the spleen cells were depleted of $CD4^+$ or $CD8^+$ T cells or $CD19^+$ B cells prior to culture. Culture supernatants of spleen cells from naïve B6 mice contained below detectable levels of IL-10. Statistically significant differences as determined by Mann–Whitney *U* test: (*) Young vs aged at the same time p.i., $P < 0.05$; (+) No depletion vs (−) $CD4^+$ or (−) $CD8^+$ cells of the same age group and time p.i., $P < 0.05$. (++) No deletion vs (−) $CD19^+$ cells of the same age group and time p.i., $P < 0.02$. (#) 2 weeks vs 4 weeks p.i. of the same age groups and under similar conditions, $P < 0.015$.

these assays and/or the amount of IL-4 produced following E55+MuLV infection, since we were able to detect both IL-4 in culture supernatant and IL-4-producing cells when aliquots of the spleen cell preparations were stimulated with Con A (data not shown). These results, however, were consistent with previous studies that showed IL-4 production by spleen cells of young E55+MuLV-infected B6 mice upon anti-CD3 mAb stimulation *in vitro* were below detectable level (Panoutsakopoulou *et al.,* 1998).

DISCUSSION

Numerous studies have demonstrated that the age-related decrease in immune function is associated with significant alterations in cytokine production (Kirschmann and Murasko, 1992; Goonewardene and Murasko, 1993; Wakikawa *et al.,* 1999; Taylor *et al.,* 1997; Hobbs *et al.,* 1991; Ernst *et al.,* 1993). However, the effect of increasing age on antigen-specific cytokine production during a virus infection has not been examined. Previously, we utilized the E55+MuLV model to investigate the relationship between decreased immune responsiveness and diminished control of primary virus infections in aged mice (ElRefaei *et al.,* 2001). Results showed that E55+MuLV infection of aged B6 mice is characterized by a considerable delay in virus clearance compared to young B6 mice. The delay in virus clearance by aged mice was associated with the lack of a T cell proliferative response to E55+MuLV and significantly lower cytotoxic T cell responses and virus-neutralizing antibody titers to E55+MuLV. In the current studies we utilized the E55+MuLV model to examine the effect of increasing age on antigen-specific cytokine production.

Three main questions were addressed within the context of virus-specific antigenic stimulation. Assuming that we would observe an alteration in cytokine production, we asked: First, is the age-associated alteration in cytokine production consistent with a shift from a type 1 to a type 2 response? Second, does this alteration represent a change in the kinetics of cytokine production with age? Third, is this alteration due to a change in the number of cells producing the cytokine, a change in the amount of cytokine being produced per cell, and/or a change in the type of cell that is producing the cytokine?

Many studies that have examined age-associated changes in cytokine production have evaluated the level of cytokine in supernatants after stimulation of lymphocytes. Therefore, our first assessment was quantitation of cytokine in the supernatants of spleen cultures at times corresponding to maximum proliferation *in vitro.* For all three cytokines that were detectable, i.e., IL-2, IL-10, and IFN-γ, the level of cytokine detected in the supernatants of spleen cells from aged mice was significantly lower than that observed in young mice. This finding was consistent with previous studies showing decreased IL-2 production upon lymphocyte stimulation (Kirschmann and Murasko, 1992; Goonewardene and Murasko, 1993; Engwerda *et al.,* 1996; Wakikawa *et al.,* 1999). It has also been postulated that increased age results in a shift from a type 1 to a type 2 cytokine response (Shearer, 1997). Consistent with this hypothesis, a decrease in IFN-γ production in response to E55+MuLV infection was also observed in aged mice.

An age-associated shift from a type 1 to a type 2 response would also predict that there would be an increase in IL-10 production with increasing age. The limited number of studies that examined IL-10 production have indeed observed an increase in IL-10 production by T cells from aged compared to young mice (Hobbs *et al.,* 1994; Spencer *et al.,* 1996). However, in contrast to our expectations, a decrease in IL-10 in the cultures from aged compared to young mice were observed. These results of the E55+MuLV system are consistent with studies of influenza stimulation of peripheral blood lymphocytes of elderly humans after influenza vaccination (Bernstein *et al.,* 1998): the level of all detectable cytokines is decreased in elderly compared to young after virus-specific stimulation.

The next question was whether or not this decrease in cytokine production merely represented a shift in the kinetics of cytokine production. The current study chose two time points for assessment of cytokine production: 2 weeks and 4 weeks p.i. Two weeks reflect the first time postinfection that there is a detectable response to E55+MuLV infection by either proliferation or cytotoxic T cell activity in young mice (Panoutsakopoulou *et al.,* 1998; ElRefaei *et al.,* 2001). The level of proliferative response in young mice increases to a maximum level at 4 weeks p.i. and this level is maintained through at least 12 weeks p.i. In aged mice, there is no significant proliferation at any time through 12 weeks p.i.; however, there is detectable cytotoxic T cell activity, which peaks at 4 weeks p.i. (ElRefaei *et al.,* 2001). Therefore, 2 weeks represent the initiation of a detectable immune response, in at least young animals, with 4 weeks being the time of maximal T cell activity of both young and aged mice. An increase in the level of all three cytokines was observed from 2 to 4 weeks p.i. in both young and aged mice. For all three cytokines, the magnitude of the increase from week 2 to week 4 was greater in aged than in young mice. For IL-2 and IFN-γ, the level of cytokine detectable in supernatants of cultures of spleen cells from young mice increased approximately 30% from 2 to 4 weeks p.i., while in aged mice it increased two- to threefold. IL-10 increased approximately twofold in cultures of spleen cells from young mice, but increased approximately fourfold from 2 to 4 weeks in cultures of spleen cells in aged mice. Regardless of the fold increase, the level of cytokines observed in cultures of spleen cells of young mice was consistently and signif-

icantly higher than the level observed in cultures of aged animals.

The fact that there was no significant proliferation detectable in the spleen cell cultures of aged mice at either 2 or 4 weeks p.i. in the presence of IL-2 production suggests that aged mice are able to synthesize, but not utilize, IL-2. Since this decreased utilization of IL-2 would result in decreased proliferation, we predicted that the number of cells producing IL-2 would be decreased in aged mice and that this may occur either independently or in conjunction with a decrease in the level of IL-2 produced per cell. To explore whether or not the decrease in the level of IL-2 was associated with a decreased number of cells producing IL-2, we utilized the ELISPOT assay. To maximize the sensitivity of the assay, we followed the protocol of previous studies (Taguchi *et al.,* 1990) and cultured spleen cells with antigen for 2 days prior to assessing cytokine secretion in the ELISPOT assay (indirect ELISPOT). Similar to the results obtained with the ELISA assay, there were fewer cells producing IL-2 in spleen cells from aged mice compared to spleen cells from young mice at both 2 and 4 weeks p.i. Intriguingly, however, there was no increase in the number of cells producing IL-2 from 2 to 4 weeks p.i. in either young or aged mice. Since the amount of IL-2 in the supernatant increased from 2 to 4 weeks p.i., the lack of an increase in the number of cells could suggest that the same number of cells are simply producing more IL-2 at 4 compared to 2 weeks p.i..

One concern with the indirect ELISPOT is that it includes a 48-h culture with antigen prior to quantitation of the cells producing the cytokine. Data from the ELISA suggested that lymphocytes were expanding during the 48-h culture since supernatants from spleen cells cultured alone or with uninfected IC21 cells contained more IL-2 than supernatants from spleen cells cultured with infected IC21 cells. If lymphocytes of aged mice fail to proliferate in response to IL-2 *in vitro,* it is possible that the difference in the number of IL-2-producing cells between aged and young mice as detected by indirect ELISPOT is larger than the difference that occurs *in vivo.* To address this possibility, we used a direct ELISPOT in which spleen cells were directly plated on the cytokine detection plate.

Results from the direct ELISPOT suggested that the extended culture prior to the indirect ELISPOT was influencing the results: the number of IL-2-producing cells detected in the direct ELISPOT was smaller at both times and in both age groups. Further, the *in vitro* culture appeared to maximally expand all virus-reactive lymphocytes, since the indirect ELISPOT had no increase from 2 to 4 weeks p.i., while the direct ELISPOT demonstrated a significant increase from 2 to 4 weeks p.i. However, the extended culture did not appear to amplify the difference between young and aged mice: there is a 40–50% decrease in the number of IL-2-producing cells in the aged mice in both assays and at both times.

Interpretation of data from all three assessments of IL-2 is consistent with the hypothesis that the decrease in IL-2 levels in culture supernatant of spleen cells from young mice following E55+MuLV-specific stimulation is due to consumption of IL-2 by T cells proliferating during the extended culture. This explanation does not appear to account for the decrease in IL-2 levels following virus-specific stimulation in aged mice; there is no significant antigen-specific proliferation in aged mice at either time as detected by ^{3}H-thymidine incorporation. However, while the overall level of proliferation of spleen cells of aged mice is not significant, there is an expansion in culture of the cells producing IL-2 in aged mice as illustrated by the higher number of IL-2 producing cells in the indirect vs direct ELISPOT. These IL-2-producing cells may be the primary cell utilizing the IL-2 that is produced. Althugh the above is the most likely explanation for the decrease in IL-2 upon *in vitro* culture with E55+MuLV-infected IC21 cells, E55+MuLV may have inhibited IL-2 production by splenic-CD4^{+} T cells from both age groups. In the closely related FV system, spleen cells from infected mice at 14–28 days p.i. suppressed production of IL-2 by normal spleen cells upon stimulation with Con A *in vitro* (Matteucci *et al.,* 1989).

The effect of aging on IFN-γ production is unclear. Some reports showed that T cells from aged B6 mice produced significantly more IFN-γ than T cells from young mice when stimulated with either anti-CD3 mAb, Con A, or phorbol myristate acetate (PMA)/ionomycin (Kirschmann and Murasko, 1992; Engwerda *et al.,* 1996; Wakikawa *et al.,* 1999; Nagelkerken *et al.,* 1991). In addition, flow cytometric analysis of spleen cells from aged mice following anti-CD3 mAb stimulation showed a significant increase in the percentage of IFN-γ-positive cells compared to spleen cells from young mice (Wakikawa *et al.,* 1999). In contrast, other studies showed significantly lower IFN-γ production by Con A or superantigen-stimulated spleen cells from aged compared to young B6 mice (Frasca *et al.,* 1997; Fujio *et al.,* 1995).

In the current studies, a delay in the production, as well as significantly lower levels, of IFN-γ were observed in aged compared to young mice upon E55+MuLV-specific stimulation of spleen cells at 2 and 4 weeks p.i. Decreased IFN-γ production was associated with decreased numbers of IFN-γ-producing cells in the spleen of aged mice. There was an increase in the number of cells producing IFN-γ in both young and aged mice at 4 compared to 2 weeks p.i. However, the percentage increase observed in the indirect ELISPOT assay did not directly relate to the increase observed in the ELISA: the level of IFN-γ in the supernatant of spleen cells from young mice increased about 30% from 2 to 4 weeks p.i., while the number of cells producing cytokines tripled. In aged mice the level of IFN-γ in culture supernatant in-

creased approximately twofold, while the number of cells producing IFN-γ increased approximately fivefold. The results suggested that either less IFN-γ is being made per cell as the immune response proceeds or that as IFN-γ is being made, it is utilized by the expanded number of cells.

Interestingly, the age-associated decrease in IFN-γ production was associated with a shift in the primary source of IFN-γ in aged mice. In young mice, splenic $CD4^+$ T cells were not only required for IFN-γ production at 2 weeks p.i., but appeared to be responsible for most of the IFN-γ production. By 4 weeks p.i., while both $CD4^+$ and $CD8^+$ T cells contributed to IFN-γ production, most of the IFN-γ was produced by $CD8^+$ T cells and about 35% of the production by $CD8^+$ T cells was independent of $CD4^+$ T cells. In contrast, depletion of $CD4^+$ T cells had little effect on IFN-γ production in aged mice, indicating that the $CD8^+$ T cells of aged mice were not only the main source of IFN-γ but also they did not require $CD4^+$ T cell help for IFN-γ production.

The age-associated alteration in IFN-γ production has not been clearly attributed to a specific subpopulation of T cells. Studies showed that both splenic $CD4^+$ (Engwerda *et al.,* 1996; Wakikawa *et al.,* 1999; Hobbs *et al.,* 1993) and $CD8^+$ (Engwerda *et al.,* 1996; Wakikawa *et al.,* 1999; Ernst *et al.,* 1993) T cells from aged B6 mice produced higher levels of IFN-γ than the corresponding cells from young mice upon anti-CD3 mAb or anti-CD3 mAb/antiCD28 mAb stimulation *in vitro.* While an age-associated increase in IFN-γ was observed in both $CD4^+$ and $CD8^+$ T cells, the difference in IFN-γ production between young and aged was much greater in $CD8^+$ T cells (Engwerda *et al.,* 1996). Flow cytometric analysis of spleen cells from aged mice also showed that the percentage of IFN-γ-positive/$CD8^+$ cells was significantly higher than the percentage of IFN-γ-positive/$CD4^+$ cells (Wakikawa *et al.,* 1999). These results are consistent with our observation that $CD8^+$ T cells are the main source of IFN-γ in aged mice.

IFN-γ is required for clearance of some virus infections. For example, B6 mice treated *in vivo* with anti-IFN-γ mAb during acute cutaneous herpes simplex virus infection failed to clear virus by 8 days p.i., whereas all control mice treated with normal rat IgG cleared virus efficiently (Smith *et al.,* 1994). Similarly, FV infection resulted in a significant increase in the production of IFN-γ, as well as an increase in the number of IFN-γ-producing $CD4^+$ and $CD8^+$ T cells at 7–12 days p.i. (Peterson *et al.,* 2000). In addition, *in vivo* treatment with anti-IFN-γ mAb during FV infection suppressed recovery from FV-induced splenomegaly until at least day 37 p.i., while all mice treated with normal anti-rat immunoglobulin (Ig) recovered from FV-induced splenomegaly by 18 days p.i. (Peterson *et al.,* 2000). While young IFN-γ knock-out B6 mice can clear E55+MuLV by 8 weeks p.i., young BALB mice do require IFN-γ for E55+MuLV clearance (Panoutsakopoulou *et al.,* 1998). Therefore, it is possible that aged B6 mice may be more similar to BALB mice and that the delayed and decreased levels of IFN-γ may be a major factor in the delayed E55+MuLV clearance in aged mice.

IL-10 plays a key regulatory role in inhibiting production of type 1 cytokines, thus downregulating cell-mediated responses. IL-10 inhibited Con A-induced IL-2 production by T cells (Ding and Shevach, 1992), and it also suppressed the ability of macrophages to stimulate Th-1 cell clones to synthesize IFN-γ (Fiorentino *et al.,* 1991). A limited number of studies showed that aging is associated with increased IL-10 production. Splenic $CD4^+$ cells from aged B6 mice that were stimulated with anti-CD3 mAb *in vitro* produced significantly higher levels of IL-10- and IL-10-specific mRNA compared to the corresponding cells from young B6 mice (Hobbs *et al.,* 1994). In other studies, spleen and peritoneal exudate cells from aged CBA mice cultured for 24 h without or with stimulation by endotoxin produced significantly higher IL-10 levels compared to cells isolated from younger mice (Spencer *et al.,* 1996). We, therefore, hypothesized that an age-related increase in IL-10 production would be associated with the decreased cell-mediated immune response to E55+MuLV resulting in delayed virus clearance in aged mice. Contrary to our expectations, spleen cells from aged mice 2 and 4 weeks p.i. produced significantly lower levels of IL-10 compared to spleen cells from young mice upon E55+MuLV-specific stimulation *in vitro.* These results demonstrate that the lack of E55+MuLV-specific T cell proliferation by spleen cells of aged B6 mice is not associated with an increase in IL-10 production.

IL-10 is produced by both T and B cells. *In vitro* lymphocyte subset depletions of spleen cells from B6 mice infected with murine gammaherpes virus 68 (MHV-68) demonstrated that both T and B cells produced IL-10 upon restimulation with virus-infected spleen cells (Sarawar *et al.,* 1996). Although our results showed that B cells were required for IL-10 production during the acute phase of E55+MuLV infection in both age groups, in young mice either $CD4^+$ or $CD8^+$ T cells were also needed for IL-10 production. In contrast, depletion of either $CD4^+$ or $CD8^+$ T cells had little effect on IL-10 levels in the culture supernatant of spleen cells from aged mice, indicating that T cells did not contribute to IL-10 production in aged B6 mice. These results provide further evidence that an alteration in $CD4^+$ T cell function occurs in aged B6 mice and that aged mice may develop alternative mechanisms for cytokine production.

Finally, our results also showed that E55+MuLV infection was associated with increased IL-10 production by spleen cells from mice of both age groups compared to spleen cells from uninfected mice. These results are consistent with other studies that demonstrated increased IL-10 production during virus infection. Intrana-

sal infection of B6 mice MHV-68 resulted in increased IL-10 production by spleen and lymph node cells after *in vitro* restimulation with virus-infected spleen cells (Sarawar *et al.,* 1996). Spleen cells from B6 mice, 1 week postinfection with a mixture of murine leukemia viruses (LP-BM5), produced higher levels of IL-10 when cultured without antigenic stimulation and when stimulated with Con A compared to spleen cells from uninfected mice (Gazzinelli *et al.,* 1992). In other studies the expression of IL-10 mRNA was upregulated in both the thymus and the lymph nodes of feline immunodeficiency virus infected cats (Liang *et al.,* 2000). The production of high levels of IL-10 during virus infection may play a role in the establishment and maintenance of persistent E55+MuLV infection.

In summary, E55+MuLV provides a unique model in which age-associated changes in the immune response to a virus infection can be explored. Since the virus infection persists for more than 2 months, an extensive evaluation of the entire range of immune responses can occur. We have previously established that aged mice demonstrate both a decrease and a delay in both antibody and cytotoxic T cell responses. The current data clearly show a decreased proliferative and cytokine response as well. Importantly, the decrease in cytokine production occurs in both type 1 (IL-2 and IFN-γ) and type 2 (IL-10) cytokines, challenging one hypothesis of immunosenscence that suggests that there is a shift from type 1 to type 2 cytokine response with age. The reduction in cytokine levels was associated with a lower number of cytokine-producing cells. In addition, there was a shift in the primary source of IFN-γ from $CD4^+$ to $CD8^+$ T cells in aged mice. Further, the dependence of IFN-γ and IL-10 production on T cell help appears to abate with increased age. The lack of significant virus-induced proliferation, decreased IL-2 production, and increased contribution of $CD8^+$ T cells to IFN-γ production support other studies that emphasize the negative impact of aging on $CD4^+$ T cell function. However, since $CD4^+$ T cells are required for reduction of E55+MuLV titers in young B6 mice and since aged B6 mice can still reduce E55+MuLV titers, this system illustrates that the aging immune system may develop compensatory mechanisms to control virus infections.

MATERIALS AND METHODS

Mice

Six-month- and 22-month-old C57Bl/6 (B6) male mice were purchased from the NIA colony of Charles River Laboratories. All mice were housed in AAALAC-certified barrier facilities at MCP Hahnemann University and were given autoclaved food and water *ad libitum.* Mice were allowed to acclimate for at least 2 weeks in our facilities prior to use. Mice demonstrating tumors of any kind were eliminated from the study.

Virus

E55+ murine leukemia virus (E55+MuLV) was originally isolated from a BALB.K spleen injected with cell-free culture supernatant from a T cell line derived from a leukemic mouse (Pozsgay *et al.,* 1989). The virus used in these studies was propagated *in vivo* by i.p. injections of immunosuppressed BALB/c mice (Wolf and Blank, 1983). All mice used in the studies were injected i.p. with 4×10^4 focus forming units (FFU) of E55+MuLV.

Antibodies

Hybridoma cells producing the monoclonal antibodies (mAb) mAb34, specific for the p15-*gag* protein, and mAb48, specific for the gp70 envelope protein (Cheseboro *et al.,* 1981), were a gift from Dr. Bruce Chesebro. Hybridoma cells producing the mAb GK1.5 (anti-CD4) and mAb 2.43 (anti-CD8) were obtained from American Type Culture Collection (ATCC, Rockville, MD). Antibodies were purified using protein A-sepharose affinity chromatography (Ey *et al.,* 1978). Rat anti-mouse IL-2 mAb (clone JES6-1A12), biotinylated rat anti-mouse IL-2 mAb (clone JES6-5H4), rat anti-mouse IL-10 mAb (clone JES5-2A5), biotinylated rat anti-mouse IL-10 mAb (clone SXC-1), rat anti-mouse IFN-γ mAb (clone R4-6A2), biotinylated rat anti-mouse IFN-γ mAb (clone XMG1.2), rat-anti-mouse B220 mAb (clone RA3-6B2), and biotinylated rat anti-mouse CD19 mAb (clone 1D3) were obtained from PharMingen (San Diego, CA).

Cell lines

The IC21 cell line is an SV40-transformed macrophage line derived from peritoneal macrophages of normal B6 mice and was obtained from ATCC. IC21 cells express class I ($H\text{-}2^b$) and class II ($I\text{-}A^b$) MHC molecules. E55+MuLV-infected IC21 cells express viral epitopes of the *env* and *gag* gene products as determined by flow cytometric staining with mAb34 and mAb48. IC21 cells were maintained in RPMI-1640 (Media Tech, VA) wtih 10% FCS (Sigma, St. Louis, MO). 1 mM L-glutamate, 100 U penicillin, 0.1 mg streptomycin, and 5×10^{-5} M 2-mercaptoethanol (complete medium).

Spleen cell preparations

Spleens were removed from individual mice and homogenized. Mononuclear cells were isolated by density centrifugation. Preparations were divided into three aliquots: One aliquot for *ex vivo* ELISPOT assays (direct ELISPOT) to determine the number of IL-2, IL-4, IL-10, and IFN-γ producing cells; the second aliquot for *in vitro* culture with E55+MuLV-infected IC21 cells to determine the virus-specific proliferative response; and the third aliquot for amplified ELISPOT assays (indirect ELISPOT) and ELISA for IL-2, IL-4, IL-10, and IFN-γ. Finally, to determine the role of T and B cells in virus-specific

proliferation and cytokine production, aliquots of the spleen cells were depleted *in vitro* of either $CD4^+$ or $CD8^+$ T cells or $CD19^+$ B cells prior to direct ELISPOT and *in vitro* culture.

T cell proliferation assay

Spleen cells from individual naïve or E55+MuLV-infected B6 mice were harvested at 2 and 4 weeks p.i. and were cultured in complete medium in 96-well plates at 1×10^5 cells/well in the presence of 2.5×10^4 naïve or E55+MuLV-infected IC21 cells. IC21 cells were treated with mitomycin-C (50 ng/ml) for 1 h at 37°C and then washed three to four times with PBS before use. Aliquots of spleen cells were depleted *in vitro* of either $CD4^+$ or $CD8^+$ T cells by treatment with antibody (GK1.5 or 2.43) and complement prior to culture. The percentage of $CD4^+$ or $CD8^+$ cells in the depleted aliquots was reduced to background levels (≤1%) as determined by flow cytometry. Splenocytes were cultured for 72 h and pulsed with 1.0 μCi [^{3}H]thymidine per well during the final 12 h of culture. Cells were harvested using a PHD cell harvester (Cambridge Technologies, Cambridge, MA) and radioactivity was counted in a liquid scintillation counter (Beckman, MD). Proliferation is expressed as net counts per minute (Net CPM) calculated as:

Net CPM = (CPM of splenocytes with IC21 cells)
− (CPM of splenocytes alone).

ELISA for cytokines

Spleen cells from individual naïve or E55+MuLV-infected B6 mice were cultured in complete medium in 96-well plates at 5×10^5 cells/well with 1×10^5 E55+MuLV-infected IC21 cells. IC21 cells were treated as described above. Aliquots of cells were depleted *in vitro* of $CD4^+$ or $CD8^+$ T cells or $CD19^+$ B cells before culture. T cell subsets were depleted as described above. B cells were depleted by treatment with anti-CD19 mAb and then by binding of the labeled cells to a magnetic column obtained from Stem Cell Technologies (Canada). Depletion of B cells was performed following the recommended protocol provided by the manufacturer. The percentage of B cells in the depleted aliquots was reduced to background levels (≤1%) as determined by flow cytometry with anti-B220 mAb.

Initial experiments established that the optimal time for detection of cytokine in culture supernatant was 40 h for IL-2 and IL-4 and 60 h for IFN-γ and IL-10. Supernatants were stored at −70°C until tested by sandwich ELISA specific for each cytokine. Cytokine ELISA was performed following the recommended protocol provided by PharMingen. Level of detection of IL-2 and IL-4 = 15 pg/ml; IFN-γ and IL-10 = 62.5 pg/ml. Results for IL-2 are expressed as pg/ml of culture supernatant. Results for IFN-γ and IL-10 are expressed as net cytokine in pg/ml (Net pg/ml) calculated as:

Net Cytokine = (Cytokine with inf. IC21 cells)
− (Cytokine with uninf. IC21 cells).

Cytokine ELISPOT assay

Spleen cells from individual naïve or E55+MuLV infected B6 mice were either used direclty in *ex vivo* ELISPOT assays or amplified by *in vitro* culture prior to ELISPOT assay. For the culture, spleen cells were incubated at 1×10^6 cells/well in a 24-well plate either alone or with 2×10^5 E55+MuLV-infected IC21 cells in complete medium at 37°C in 5% CO_2 for 48 h (the time established as optimal in preliminary experiments). Aliquots of the spleen cells were depleted *in vitro* of $CD4^+$ or $CD8^+$ T cells prior to *ex vivo* ELISPOT assays or amplification culture described above. After culture, cells were washed, counted, resuspended in complete medium, and used in the ELISPOT assay (Klinmann and Nutman, 1995). Briefly, Immunlon II plates (Dynatech, Chantilly, VA) were coated overnight at 4°C with anti-mouse capture mAb (either 2 μg/ml of anti-IL-2 or 5 μg/ml of anti-IFN-γ in PBS). Plates were washed three times with PBS/Tween-20 (0.5%) and blocked with complete media for 2 h at 37°C. Spleen cells of both *ex vivo* and *in vitro* culture were added to individual wells at twofold serial dilutions (4×10^5–1×10^5 cells/well) and incubated for 20 h at 37°C. Plates were then washed three times with PBS followed by three additional times with PBS/Tween-20 (0.5%). Biotinylated detection mAb for both IL-2 and IFN-γ was diluted to 2 μg/ml in PBS/Tween-20 (0.5%) and was added to the wells. Plates were incubated overnight at 4°C and then washed three times with PBS followed by three additional times with PBS/Tween-20 (0.5%). Strepavidin-akaline phosphatase (Sigma), diluted in PBS/Tween-20 and 5% FCS, was added and the plates were incubated for 1 h at 37°C. Plates were washed five times with PBS/Tween-20 (0.5%) and then with BCIP (5-bromo-4-chloro-3-indolyl phosphate) (Sigma) at 1 mg/ml in AMP buffer (0.15 M 2-amino-2-methyl-1-propanol) containing 0.6% agarose was added to the wells. The plates were stored at room temperature until the agarose set and were scored the next day. Cytokine-producing cells were counted with the aid of a dissecting microscope (SZH Zoom stereo microscope, Olympus, Lake Success, NY). All scoring was performed by a single investigator in a coded manner. Results for IL-2 are expressed as the number of cytokine-producing cells per 10^6 cells. Results for IFN-γ are expressed as net IFN-γ producing cells per 10^6 cells calculated as:

Net IFN-γ cells = (IFN-γ cells with inf. IC21)
− (IFN-γ cells with uninf. IC21 cells).

Statistical analysis

All statistics were performed using JMP software version 3.2.6 (SAS Institute). Statistical significance (except for T cell proliferation) was determined using Mann–Whitney *U* test. Unpaired Student's *t* test was used to determine statistical significance for T cell proliferation.

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